

Ellman's Reagent: Interference in Mercapto Group Determination, with Special Reference to Cigarette Smoke

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Cyanide, hydrogen sulphite and sulphide interfere in the determination of mercapto groups with Ellman's reagent [5,5'-dithiobis-(2-nitrobenzoic acid)] by nucleophilic substitution of the reagent. Such reactions produce significant analytical errors under certain conditions. Some saturated and unsaturated aliphatic aldehydes also interfere in the determination by formation of less reactive addition products with the mercapto compounds. When both types of compounds are present concurrently, as in cigarette smoke and related products, the aldehydes and the thionucleophilic reagents may react with each other and with other smoke components in a series of complex reactions. The degree of de-activation of mercapto groups by cigarette smoke and related products can be determined with Ellman's reagent only under carefully controlled conditions.

ELLMAN'S reagent [5,5'-dithiobis-(2-nitrobenzoic acid)] (DTNB) is used extensively for the determination of mercapto groups in enzymes and proteins.^{1,2} The reagent is usually used under a wide variety of experimental conditions with satisfactory results. However, when applied in this laboratory to a recent problem involving the mechanisms of enzymatic inhibition by cigarette smoke, extensive analytical interferences were observed. Past work on this inhibition had shown that such mechanisms are complex³ and may include de-activation of mercapto groups of enzymes, as enzymatic inactivation is reduced in the presence of cysteine.^{3 to 9} In attempting to differentiate the inhibitory mechanisms it was desired to determine analytically the degree of mercapto group inactivation during inhibition. The results showed that considerable interference in such determinations occurs when DTNB is used. The interfering compounds are present in cigarette smoke and some natural products, as well as in air pollution and industrial wastes. Recognition of these interferences is important in studies involving mercapto group determinations on such products.

EXPERIMENTAL

CIGARETTE SMOKE—

Commercial 85-mm cigarettes with a multiple cellulose acetate - activated carbon filter were used. In some experiments the activated carbon was removed from the filter or the entire filter was removed from the cigarettes. All cigarettes were mechanically smoked under the following conditions: puff volume, 35 ml; puff duration, 2 s; and puff interval, 1 minute⁻¹. Whole smoke was collected by passage through 0.067 M phosphate buffer (pH 7.4) in a gas scrubber. The vapour phase of smoke was similarly collected after passing the mainstream smoke through a compressed fibreglass (Cambridge) filter to remove particulate matter. The smoking procedure was adjusted so that the smoke from 1.0 cigarette was collected in 1.0 ml of buffer.

REAGENTS—

5,5'-Dithiobis-(2-nitrobenzoic acid) (DTNB)—DTNB (supplied by K. & K. Laboratories) was recrystallised three times from glacial acetic acid, and a 0.01 M stock solution in 0.05M phosphate buffer (pH 8.0) was prepared. Two millilitres of this stock solution were diluted with distilled water to 100 ml for use.

Working buffer—This was prepared by diluting 60 ml of 0.05 M buffer (pH 8.0) to 100 ml. These solutions give the same final concentration of buffer and DTNB as used in the original method.²

Glutathione and cysteamine (2-aminoethanethiol) (each obtained from Calbiochem) were used at 10^{-4} M concentration. Known constituents of cigarette smoke were prepared as aqueous stock solutions containing concentrations equivalent to the amounts found in 100 cigarettes ml^{-1} of solution. Other compounds were studied at a concentration of 10^{-4} M.

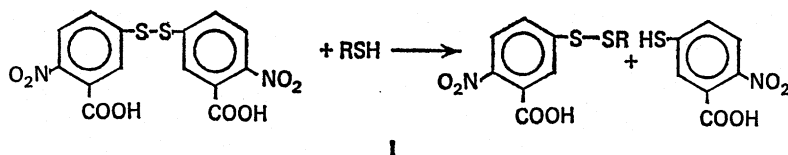
ANALYTICAL METHODS

All reactions were performed in 1-cm quartz cuvettes (capacity 3.0 ml). To 1.0 ml of the working buffer was added 1.0 ml of the solution of the mercapto group containing compound. After 30 s, 1.0 ml of DTNB solution was added, and the absorbance was determined after a reaction time of 30 s or longer. No working buffer was added when smoke solutions or solutions of smoke constituents were used. Glutathione standards were used with each determination. In certain experiments, the order of addition or the interval of time between additions of the components was changed. All readings were made at 412 nm (with a DTNB blank) with a Perkin-Elmer 202 spectrophotometer connected to a Moseley 7000-A X-Y recorder.*

* Mention of a specific commercial product does not imply endorsement by the Department over others not named.

RESULTS AND DISCUSSION

Analytical determination of mercapto groups with DTNB is based on scission of the disulphide linkage to form the chromogen, 5-mercapto-2-nitrobenzoic acid, which absorbs at 412 nm, I. To determine the rôle of mercapto inactivation in the over-all enzymatic



inhibition by cigarette smoke, it was originally decided to compare the rate of such inhibition with the rate of mercapto disappearance by using a compound of simple structure containing a mercapto group rather than an enzyme containing this group. With cysteamine, some smoke solutions reduced the apparent mercapto content, as expected, but the quantitative values varied widely, depending on the experimental conditions.

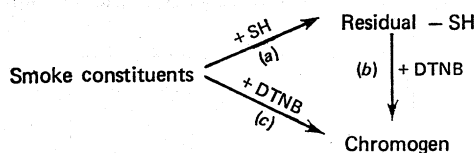
TABLE I
REACTION OF VAPOUR PHASE OF CIGARETTE SMOKE WITH CYSTEAMINE AND
ELLMAN'S REAGENT (DTNB)

Order of addition*			Reaction time, s*	Initial absorbance (<i>A</i>)	ΔA
1	2	3			
Vapour phase	DTNB	Cysteamine	30	0.240	0.070
Vapour phase	Cysteamine	DTNB	15	0.180	0.095
Vapour phase	Cysteamine	DTNB	30	0.130	0.120
Vapour phase	Cysteamine	DTNB	60	0.055	0.150
Buffer	Cysteamine	DTNB	30	0.240	0.0
Vapour phase	Buffer	DTNB	30	0.0	0.180
Cysteamine	DTNB	Vapour phase	30	0.240	0.085

* Components 1 and 2 reacted for the time indicated, then component 3 was added and the initial absorbance (*A*) read 30 s later and at intervals up to 60 minutes. ΔA = total increase in absorbance from 30 s to 60 minutes.

Table I illustrates the patterns obtained. In these experiments, reaction time and the order of addition of DTNB, the vapour phase of cigarette smoke and cysteamine were varied. Cysteamine alone reacts rapidly with DTNB. The maximum development of chromogen is attained in 30 s and no further increase in absorbance develops up to 60 minutes. In the

absence of cysteamine, the vapour phase of cigarette smoke also reacts with DTNB but at a slow rate, giving no absorbance at 30 s but significant absorption at 60 minutes; reaction times of 60 minutes are frequently used with DTNB. These results show that two independent groups of reactions are proceeding with DTNB: one is rapid and involves scission of the reagent by the residual mercapto groups [reactions (a) and (b), II]; and the other is slow and involves direct scission of DTNB by one or more compounds in smoke [reaction (c), II]. When the order of addition is 1, vapour phase, 2, DTNB and 3, cysteamine, or the reverse, the same absorbance is attained in 30 s and rapid reactions (a) and (b) are primarily being read. If vapour phase and mercapto groups are permitted to react for 15, 30 and 60 s before addition of the reagent, the subsequent absorbances at 30 s show an inverse relationship with the three reaction times, indicating that the vapour phase is reacting with the mercapto groups in the expected manner via reactions (a) and (b) in II. With times from 30 s to 60 minutes further increases in absorbance occur resulting from slow reaction (c), but these increases are directly related to the reaction time between vapour phase and cysteamine. Thus, false values can be obtained for mercapto-group reduction if long reaction times with DTNB are used under these conditions.



II

Known vapour-phase constituents were tested for reactivity with DTNB alone and after prior exposure to cysteamine to determine the specific components possibly contributing to the interference. The constituents were examined at concentrations equivalent to those found in the smoke from one cigarette.^{10,11} The results are shown in Table II. Hydrogen

TABLE II
REACTIVITIES OF SOME CIGARETTE SMOKE COMPONENTS WITH CYSTEAMINE OR
ELLMAN'S REAGENT (DTNB)

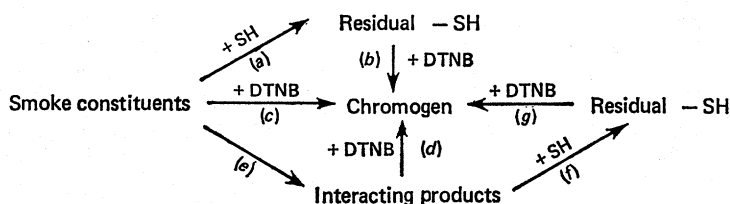
Component*	Level, μ moles	Reactivity†	
		With DTNB	With cysteamine
Hydrogen cyanide.. ..	8.50	+	0
Hydrogen sulphide	1.32	+	0
Sulphur dioxide	0.47	+	0
Acetaldehyde	16.8	0	+
Acrolein	1.64	0	+
Crotonaldehyde	0.21	0	+
Formaldehyde	1.76	0	+

* Nicotine, phenol, acetone, methanol, furfural, pyridine, acetonitrile, nicotinonitrile and oxides of nitrogen did not react.

† Aqueous solutions of components mixed with DTNB or with cysteamine for 30s followed by DTNB, and absorbance (A) at 412 nm read 30 s after addition of DTNB.

0 = No reaction. + = Reaction.

cyanide, hydrogen sulphide and sulphur dioxide react with the reagent alone and give chromogenic absorbance even after 30 s. Formaldehyde, acetaldehyde, acrolein and crotonaldehyde do not react with DTNB in 30 s but react with cysteamine giving reductions in apparent mercapto content within that time period. These results may appear to be anomalous as the reactions between total vapour phase and DTNB do not give absorbance at 30 s (Table I), but some of the above smoke components present in the same concentration as found in the vapour phase generate a chromogen under these conditions. This indicates that the vapour-phase constituents themselves are interacting and that the system may be more accurately represented by III. Further evidence of the interactions is shown in Fig. 1.



III

As vapour phase does not react with DTNB in 30 s (Table I), the reaction rates, k_c and k_d , are slower than the rates for the other reactions and the over-all absorbance at 30 s is, therefore, a reflection of k_a , k_b , k_e , k_f and k_g , all of which are related to the degree of mercapto-group disappearance. The variations in 30-s readings with single compounds and their combinations in Fig. 1 show that k_e and k_f may be slow or rapid, depending on the compounds.

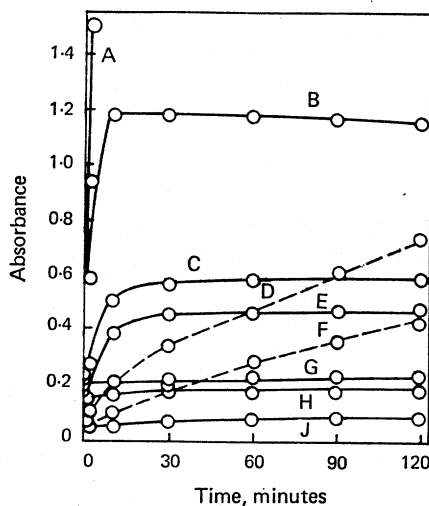


Fig. 1. Effect of vapour-phase constituents of cigarette smoke on the measurements of the mercapto content of cysteamine by using 5,5'-dithiobis-(2-nitrobenzoic acid). Cysteamine (50 μ moles) + indicated constituents in concentrations shown in Table II were allowed to react for 30 s, 200 μ moles of DTNB were added and the absorbance was read after 30 s. A, cyanide, hydrosulphide, cyanide + formaldehyde + acetaldehyde or cyanide + acrolein + crotonaldehyde; B, hydrogen sulphite; C, hydrosulphide + acrolein + crotonaldehyde; D, hydrosulphide + formaldehyde + acetaldehyde; E, hydrogen sulphite + acrolein + crotonaldehyde; F, hydrogen sulphite + formaldehyde + acetaldehyde; G, none; H, acrolein + crotonaldehyde; and J, formaldehyde + acetaldehyde.

The two unsaturated aldehydes tend to react more rapidly with hydrogen sulphide (as hydrosulphide) and sulphur dioxide (as hydrogen sulphite) than the two saturated aldehydes. Apparently, reactions between hydrogen cyanide and the aldehydes occur very rapidly, if at all.

In general, it appears that DTNB can be used as an analytical reagent for mercapto-group determination in the presence of the interfering substances in cigarette smoke if the reaction time is kept very brief. This is in contrast to analyses for mercapto groups in proteins, which require longer reaction periods, *e.g.*, 15 to 30 minutes.¹² By using a reaction time of 30 s in the present analytical system, the following percentage reductions in mercapto groups of cysteamine were obtained: whole smoke from non-filter cigarettes, 75; vapour phase of smoke from such cigarettes, 60; whole smoke from cigarettes with the multiple activated carbon - cellulose acetate filter, 20; and aged (24 hours) whole smoke from cigarettes with a cellulose acetate filter, 20. In general, these results parallel previously reported findings on the degrees of enzymatic inhibition by smoke and smoke phases^{3,4} and indicate that mercapto-group disappearance is occurring as a major inhibitory mechanism.

The slow interfering reactions between smoke constituents and DTNB are probably similar in mechanism to the scission of the disulphide bond by the mercapto group, *i.e.*, S_N2 .¹³ Among the groups known to cleave disulphide bonds are cyanide, hydroxide, hydrogen sulphite, hydrosulphide, thiocyanate, azide and iodide.¹³ In addition to hydrogen cyanide and hydrogen sulphide, thiocyanic acid and thiocyanogen have been reported in cigarette smoke⁹ and evidence for the presence of sulphur dioxide has been obtained.¹⁰ The secondary amines, piperidine and pyrrolidine, are also smoke constituents⁹ and have been reported to cleave the disulphide bonds when present as the free amines.¹⁴ These two bases were tested for reaction with DTNB but did not react at the pH used (pH 8.0); at this pH, both compounds exist predominantly as the quaternary ammonium salts. Some reaction is observed when the bases are predominantly in the un-ionised state (pH 12.0), but this reactivity may be caused in part by hydroxide ion, which also reacts with disulphide.¹⁵

Several other thionucleophiles that are not smoke constituents were examined for reaction with DTNB; sodium thiosulphate and sodium dithionite were found to react positively. Dithionite reacts almost instantaneously to release the chromogen by a nucleophilic displacement but then the yellow colour gradually disappears, presumably as a result of further reaction of an unknown nature. Reductants such as hydroquinone or sodium nitrite do not react with DTNB. When high-voltage electrophoresis at pH 6.4 was used to separate the reaction products of DTNB with cyanide or smoke solutions, anomalous results were noted, including the presence of an "eyebrow" near the DTNB that was more negatively charged than the parent compound. These products were not identified but similar findings have been reported by others.¹⁶

The aldehydes in smoke appear to play a special rôle in the disappearance of mercapto groups and in the interference in the analytical determination of apparent mercapto content with DTNB with extended reaction times. Reactions between mercapto groups and aldehydes are well known¹⁷ and, in one instance, a cyclic addition product, formed by the reaction between cysteine and the acetaldehyde in cigarette smoke, has been identified as 2-methyl-L-thiazolidine-4-carboxylic acid.⁸ The reaction products between aldehydes and thionucleophilic reagents in smoke produce some kinetic differences in the subsequent reaction of such mixtures with mercapto groups or with DTNB. In several instances, a change from a first-order to a pseudo zero-order reaction was observed. Such a shift may be the result of the formation of complexes such as cyanohydrins; the latter may dissociate to yield cyanide, which will react with DTNB at an apparently altered reaction rate as the dissociation rate may be the controlling factor. This type of interaction may help to explain some of the findings on the inactivation of enzymes by tobacco smoke,^{3 to 9} as well as some of the anomalous results reported for the ciliastatic activity of cigarette smoke solutions. Several workers have reported that hydrogen cyanide, ammonia, formaldehyde, acrolein and nitrogen dioxide have appreciable ciliary-depressant activity when tested individually.^{18,19} Although removal of most of the hydrogen cyanide by cigarette filters reduced ciliastasis, a similar effect was not observed when acrolein and acetaldehyde were reduced, and the authors suggested that acrolein, a strong ciliastat, was de-activated in the presence of smoke.¹⁹ It is evident that the biological and chemical reactivities of the individual compounds found in smoke may be quite different when the constituents are present in the smoke mixture because of complex formation and interactions.

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